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O-Glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*)

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Abstract

Herbicide safeners manipulate herbicide selectivity by enhancing the activities of detoxifying enzymes, such as glutathione transferases (GSTs) and cytochrome P450 mono-oxygenases (CYPs) in cereal crops. As part of a study examining the importance of *O*-glucosyltransferases (OGTs) in pesticide metabolism in hexaploid bread wheat (*Triticum aestivum* L.), seedlings were grown in the presence of dichlormid, a safener used in maize and cloquintocet mexyl, a wheat safener. The efficacy of the treatments was confirmed by monitoring changes in the abundance of phi and tau class GSTs. OGT activities in the root and shoot tissue were assayed using phenolics of natural and xenobiotic origin to determine if they were enhanced by safeners. Cloquintocet mexyl selectively increased OGT activities toward xenobiotics (4-nitrophenol and 2,4,5-trichlorophenol) and flavonoids, (quercetin, luteolin, genistein and coumestrol) in both the roots and shoots. However, OGT activity towards simple phenols and phenylpropanoids was not enhanced by cloquintocet mexyl. Dichlormid was a much weaker enhancer of OGT activity, with the same subset of OGT activities increased as determined with cloquintocet mexyl, but with the effect being largely restricted to the roots. OGT activities were also determined in black-grass (*Alopecurus myosuroides* L.), an agronomically important weed in wheat. Two populations of black-grass differing in their sensitivity to herbicides were analysed. The population Peldon, which is resistant to multiple classes of herbicides due in part to the elevated expression of CYPs and GSTs active in herbicide detoxification, contained higher OGT activities than herbicide sensitive black-grass. Unlike wheat, treatment with cloquintocet mexyl or dichlormid, had no effect on OGT activities in either black-grass population.

Keywords: *Triticum aestivum* (Poaceae); *Alopecurus myosuroides* (Poaceae); *O*-Glucosyltransferase; Herbicide safeners; Flavonoids; Glutathione transferases; Xenobiotics; Chlorinated phenols; Herbicide resistance

1. Introduction

Plants are able to metabolise a diverse range of xenobiotics, notably organic pollutants and pesticides, using enzymes that are normally used in the synthesis and processing of endogenous natural products (Cole and Edwards, 2000). Species-dependent variations in such secondary metabolism determine the rates and routes of detoxification of pesticides in crops and weeds, which is a primary determinant of herbicide selectivity (Owen, 2000). A good example of relative rates of detoxification determining herbicide selectivity is seen in wheat (*Triticum aestivum* L.) and competing weeds, such as black-grass (*Alopecurus myosuroides* L.). Herbicides that are used as selective graminicides in wheat, such as the aryloxyphenoxypropionates and phenylureas, are more readily detoxified in the crop than in competing grass weeds (Owen, 2000). Significantly, the long-term use of these herbicides has helped select for populations of weeds, especially black-grass, which have developed resistance to multiple classes of herbicides due to an enhanced ability to detoxify graminicides (Hall et al., 1997).

Different herbicides undergo different routes of detoxification in wheat. A small number of herbicides used in wheat are detoxified by glutathione conjugation

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mediated by glutathione transferases (GSTs, EC 2.5.1.18) including the aryloxyphenoxypropionate fenoxaprop ethyl, the sulphonyl urea flupyrsulphuron methyl and the chloroacetamide dimethenamid (reviewed by Edwards and Dixon, 2000). However, the most commonly observed route of detoxification of herbicides in wheat involves an initial hydroxylation, typically mediated by a cytochrome P450 mixed function oxidase (CYP), followed by *O*-glucosylation of the oxidised metabolite by UDP-glucose-dependent *O*-glucosyltransferases (OGTs, EC 2.4.1.-). The latter reaction normally completes the detoxification of the herbicide and targets the conjugated metabolite for vacuolar sequestration (Cole and Edwards, 2000). However, despite the importance of glucose conjugation in pesticide metabolism, little is known concerning the OGTs involved in xenobiotic detoxification in wheat. An OGT active in conjugating pentachlorophenol was partially characterised as a 43 kDa enzyme in wheat cell suspension cultures (Schmitt et al., 1985). In addition, a *N*-glucosyltransferase (NGT) active toward 3,4-dichloroaniline was identified in crude extracts from wheat cultures and plants (Schmidt et al., 1995). Although wheat is known to contain a diverse range of glycosides of natural products including flavonoids (Estiarte et al., 1999) and cyclic hydroxamates (Nakagawa et al., 1995), relatively little is known about the OGTs which catalyse these reactions. An exception is the recent characterisation of two OGT isoenzymes in wheat seedlings with molecular masses in the range 47–49 kDa which had high activity toward the hydroxamate 2,4,-dihydroxy-7-methoxy-1,4,-benxoxazin-3-one (Sue et al., 2000).

As a prelude to characterising the OGTs involved in pesticide metabolism in wheat and competing weeds in more detail, we are interested in establishing how OGTs are regulated as compared with other xenobiotic detoxifying enzymes, notably CYPs and GSTs. In wheat, it is well established that CYPs and GSTs that have roles in herbicide detoxification are induced following treatment with herbicide safeners, compounds which increase herbicide tolerance in cereal crops by enhancing their detoxification capacity (Davies and Caseley, 1999). We were, therefore, interested in determining if herbicide safeners also enhanced OGT activities toward xenobiotics and natural products. In addition, CYPs and GSTs are implicated in metabolism-based resistance to multiple herbicides in grass weeds such as black-grass (Hall et al., 1997). Thus, the black-grass population isolated from Peldon in the UK, which shows cross resistance to phenylurea and aryloxyphenoxypropionate herbicides, contains higher activities of CYPs (Hyde et al., 1996) and higher levels of specific GSTs (Cummins et al., 1999), than herbicide-sensitive black-grass. We were, therefore, interested in determining if herbicide cross–resistance in the Peldon black-grass was also associated with enhanced expression of OGT activities.

2. Results

2.1. OGT activities in wheat

OGT activities were determined by incubating $(NH_4)_2SO_4$ precipitated proteins of crude protein extracts with a range of phenolic compounds of natural or synthetic origin in the presence of UDP-[U-^{14}C-glucose]. Conjugate formation was then monitored by partitioning the [^{14}C-glucosylated]-derivatives into organic solvent and quantifying by liquid scintillation counting, with the radioactive conjugates analysed by HPLC (Parry and Edwards, 1994). To optimise conditions for assaying OGT activity, the model xenobiotic 2,3,5-trichlorophenol and the flavonol quercetin (Fig. 1) were incubated with UDP-[^{14}C-glucose] and crude preparations from wheat shoots and roots, under a variety of assay conditions. In both cases, when the products of the reaction were analysed by radio-HPLC, single peaks of activity were identified which were not observed in the absence of the aglycone substrate (data not shown). These analyses confirmed that OGT-mediated conjugation resulted in the formation of a single major product in each case. With both substrates, optimal activity was determined between pH 7.5 and 8.5 using Tris–HCl buffer (data not shown). In all subsequent assays OGT activity was determined at pH 8.0. Extraction and assay conditions were also optimised with respect to the presence of reducing agent and salts. Wheat shoots were extracted in the absence of thiol reducing agent and then assayed for OGT activity with and without the inclusion of 2 mM dithiothreitol (DTT). The presence of DTT caused a 10-fold increase in OGT activity toward quercetin and a 6-fold increase in activity toward 2,3,5-trichlorophenol. Additions of more DTT did not enhance further activity. In all subsequent extractions and assays of the OGTs 2 mM DTT was included in the medium. Similarly, the wheat OGTs active towards cyclic hydroxamates were more active in the presence of thiol agents such as 2-mercaptoethanol (Sue et al., 2000). The OGT activities toward quercetin and 2,3,5-trichlorophenol were also sensitive to inhibition by salts and divalent cations. The presence of 0.5 M NaCl or 0.5 M $(NH_4)_2SO_4$ reduced OGT activity toward both 2,3,5,-trichlorophenol and quercetin by 90% and 80% respectively. In contrast, while 5 mM $MgCl_2$ had no effect on either activity, the presence of 2 mM $MnCl_2$ reduced OGT activity toward quercetin by 90%, while having no effect on the conjugation of 2,3,5-trichlorophenol. The differential inhibition of the two OGT activities by $MnCl_2$ gave the first clear indication that different enzymes catalysed the conjugation reactions with the flavonol and chlorinated phenol. The wheat OGT, which conjugates cyclic hydroxamates was also sensitive to inhibition by Mn^{++} ions (Sue et al., 2000). Using shoot extracts, with either quercetin or

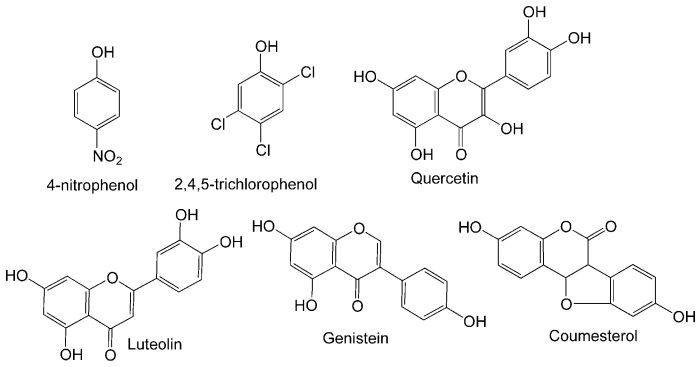


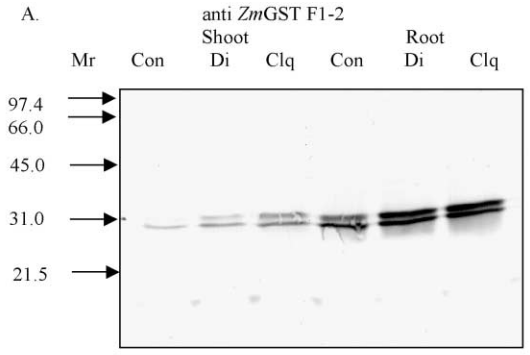
Fig. 1. Optimal OGT substrates of those tested in wheat.

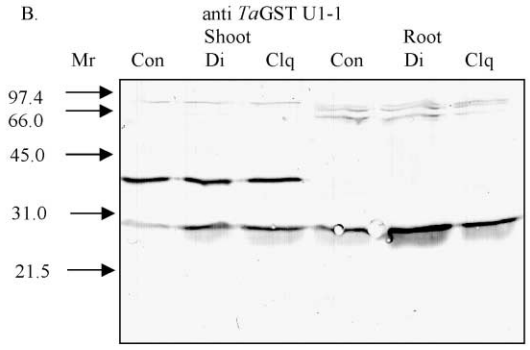
2,3,5,-trichlorophenol as substrates, product formation was directly proportional to protein content up to 200 μg protein per assay. With the root extracts, this relationship was linear up to 40 μg protein per assay. For all further comparative assays, assays of shoot extracts contained 100 μg protein and assays of roots 40 μg protein per assay respectively. Under these conditions it was established that product formation was linear with respect to incubation time up to 30 min. In all subsequent assays it was ensured that final product formation fell within the limits of strict dependence on protein content and incubation time determined above. In this way, the OGT activities per unit protein could be directly compared in different plant extracts.

2.2. OGT activities in safener treated wheat

A range of phenolics and carboxylic acids of natural and synthetic origin representing a diverse range of commonly encountered glucose acceptors (Fig. 1) were assayed as OGT substrates using crude shoot and root extracts from wheat. In shoot extracts, after correcting for the background conjugation rate (0.36 pmol [^{14}C-glucose]-conjugate formed min^{-1} mg^{-1} protein), the optimal substrates were determined to be the synthetic phenolics 2,4,5-trichlorophenol (2.55 pmol min^{-1} mg^{-1}) and 4-nitrophenol (1.37 pmol min^{-1} mg^{-1}), the flavonol quercetin (2.58 pmol min^{-1} mg^{-1}), the flavone luteolin (1.03 pmol min^{-1} mg^{-1}), the anthocyanin cyanidin (1.02 pmol min^{-1} mg^{-1}) and the isoflavones coumestrol (1.49 pmol min^{-1} mg^{-1}) and genistein (0.85 pmol min^{-1} mg^{-1}). A similar range of activities to these substrates was identified in the root extracts, along with similar specific activities in each case. Using crude extracts from either root or shoot, negligible activity was determined with the natural products benzoic acid, salicylic acid, umbelliferone, 4-hydroxycinnamic acid, caffeic acid, ferulic acid, coniferyl alcohol, isoliquirtigenin, or naringenin. Similarly, the xenobiotics phenol, 4-hydroxyphenylpyruvic acid, 2,4-dichlorophenoxyacetic acid and the herbicides chloramben and picloram were also poor substrates of wheat OGTs. From these studies 2,4,5-trichlorophenol and 4-nitrophenol, luteolin, quercetin, genistein and coumestrol were selected as model substrates for further characterisation of the regulation of OGT activity in wheat, as they represented distinct phenolic chemistries of both synthetic and natural origins.

In view of the known induction of CYPs and GSTs in wheat following treatment with herbicide safeners (Davies and Caseley, 1999), it was of interest to determine whether or not OGT activities were also affected by exposure to these compounds. Two safeners, dichlormid and cloquintocet mexyl, were selected for study. Dichlormid (*N,N*-diallyldichloroacetamide) is used to enhance tolerance to chloroacetanilide herbicides in maize due to enhancement of multiple herbicide-detoxifying GSTs (Dixon et al., 1998). Cloquintocet mexyl, is used to safen the aryloxyphenoxypropionate herbicide clodinafop propargyl in wheat, exerting its protective effect primarily by increasing the rate of CYP-mediated detoxification of the herbicide (Kreuz et al., 1991). Wheat seedlings were given a constant exposure to the safeners for 10 d and then assayed for the enhancement of GST polypeptides and GST activity to confirm safening (Edwards and Cole, 1996; Cummins et al., 1997). Using the model GST substrate 1-chloro-2,4-dinitrobenzene as substrate, both safeners enhanced GST activity in the roots and shoots, with cloquintocet mexyl being more effective than dichlormid in the roots (Fig. 2). As determined by immunoblotting following SDS-PAGE with an antiserum raised to *Zm*GSTF1-2, an immunoreactive polypeptide was recognised in both roots and shoots of untreated wheat plants (Fig. 2A). The maize phi GST *Zm*GSTGF1-2 used to raise this antiserum is specifically induced by safeners such as dichlormid in maize foliage (Dixon et al., 1998). This induction is predominantly due to the increased expression of the *Zm*GSTF2 subunit (Jepson et al., 1994). Previous studies have demonstrated that the anti-*Zm*GSTF1-2 antiserum recognises safener-inducible phi GSTs in wheat (Cummins et al., 1997). The antiserum raised against the wheat tau class *Ta*GSTU1-1, the major constitutively expressed GST in wheat, recognised a 25 kDa polypeptide in both the





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Organ	Control	Dichlormid	Cloquintocet mexyl
	GST activity nkat mg^{-1} protein (mean ± SE)		
Root	8.2 ± 0.1	11.8 ± 0.3	13.5 ± 0.6
Shoot	2.3 ± 0.0	3.9 ± 0.3	4.2 ± 0.2

Fig. 2. Demonstration of safener enhancement of GST polypeptides in the roots and shoots of 10 day old wheat seedlings treated with either 0.1% acetone (Con), 0.1 mg ml^{-1} dichlormid (Di) or 0.1 mg ml^{-1} cloquintocet mexyl (Clq). Following SDS PAGE and electroblotting, immunoreactive GST polypeptides were identified using antisera raised to (A) the safener-inducible phi-class maize *Zm*GST1-2 and (B) the constitutive tau-class wheat *Ta*GSTU1-1. For reference, molecular markers (Mr) are shown in kDa. C. GST activities towards 1-chloro-2,4-dinitrobenzene in the respective plant extracts (means±s.d., $n=3$).

roots and shoots of untreated plants (Fig. 2B). This polypeptide corresponded to the *Ta*GSTU1 subunit (Cummins et al., 1997). In addition, the antiserum also recognised unrelated polypeptides of greater molecular mass, notably a 40 kDa entity in the shoots (Fig. 2B). Treatment with dichlormid or cloquintocet mexyl selectively increased the abundance of the *Ta*GSTU1 polypeptide, most notably in the roots. Previous studies have shown that the expression of GSTs containing the *Ta*GSTU1 subunit increase in safener-treated wheat (Cummins et al., 1997). These studies therefore demonstrated that the safeners had caused a selective induction of GST polypeptides in wheat (Edwards and Dixon, 2000).

The wheat shoots and roots were then assayed for changes in OGT activity following treatment with either dichlormid or cloquintocet mexyl. Treatment of the shoots with the wheat safener cloquintocet mexyl, resulted in significant increases in OGT specific activity toward 2,4,5,-trichlorophenol, quercetin, luteolin, genistein and coumestrol (Fig. 3). In contrast, the maize safener dichlormid was less effective, giving only minor enhancement in OGT activity toward 2,4,5-trichlorophenol in shoot extracts.

Cloquintocet mexyl increased the same OGT activities in root extracts as were enhanced by this safener in shoots. Similarly, dichlormid also enhanced OGT activity toward 2,4,5-trichlorophenol, quercetin, luteolin and coumestrol in the root extracts. These results showed that both safeners up-regulated OGT activities toward both natural products and synthetic phenols in wheat, but that the enhancing effect with dichlormid was limited to the site of safener application, namely the roots.

To examine the enhancement of OGTs by safeners in more detail, wheat seedlings treated with cloquintocet mexyl were extracted and crude protein preparations applied to a DEAE anion exchange column. The profiles of eluting OGT activity toward 2,4,5-trichlorophenol, quercetin, luteolin and coumestrol were compared with that determined with extracts from control plants (Fig. 4). In control plants, all the OGT activities eluted as broad peaks along with the majority of the protein (Fig. 4A). When an identical amount of protein from the cloquintocet mexyl treated wheat shoots was applied to the column, the OGT activities eluted with similar retentions, though the enzyme activity present in the respective fractions were clearly higher with all substrates tested (Fig. 4B).

2.3. OGT activities in herbicide-resistant and herbicide-sensitive black-grass

OGT activities toward phenolics were also determined in the shoots of herbicide-susceptible (wild type) and herbicide-resistant (Peldon) black-grass (Table 1). As determined in wheat, negligible OGT activities were determined with simple phenols, benzoic acids or phenylpropanoids as substrates in either population. In the wild-type black-grass, the specific OGT activity toward the chlorinated phenols and flavonoids was very similar to that determined in untreated wheat shoots. In the herbicide-resistant black-grass, after correcting for the background conjugation rate, OGT activities were three fold higher than determined in the wild type black-grass when tested against the xenobiotics and luteolin, two fold higher with quercetin and six times higher with genistein and coumestrol. These results suggested that there was a selective enhancement of OGT activities in the herbicide-resistant population, particularly of those enzymes with activity toward isoflavones.

Although cloquintocet mexyl is known to have no herbicide safening activity in black-grass (Kreuz et al., 1991), other safeners have been shown to enhance herbicide detoxifying enzymes such as GSTs in herbicide-susceptible populations of this weed (Cummins et al.,

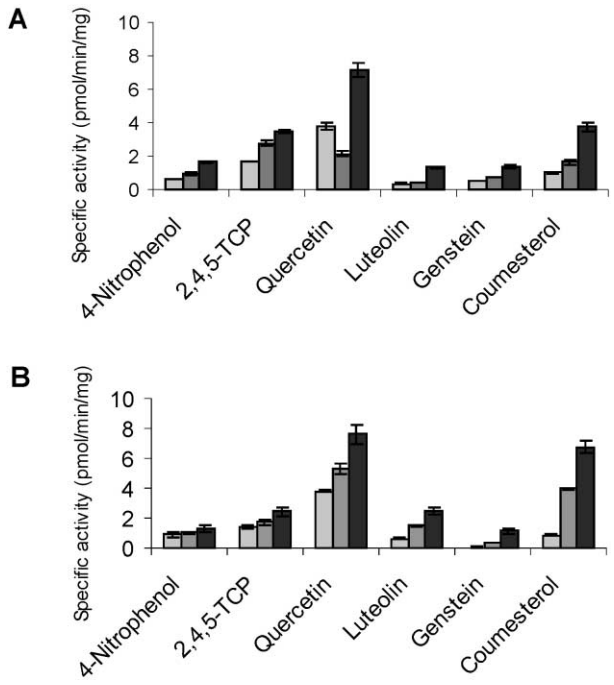

Fig. 3. OGT activities in 10 day old wheat shoots (A) and roots (B) treated with 0.1% (v/v) acetone (□), 0.1 mg ml^{-1} dichlormid (▒), 0.1 mg ml^{-1} cloquintocet mexyl (■). Values represent the means of duplicate determinations with the error bars showing the extent of variation in the replicates. All values are corrected for the background conjugation rate determined in the absence of added aglycones.

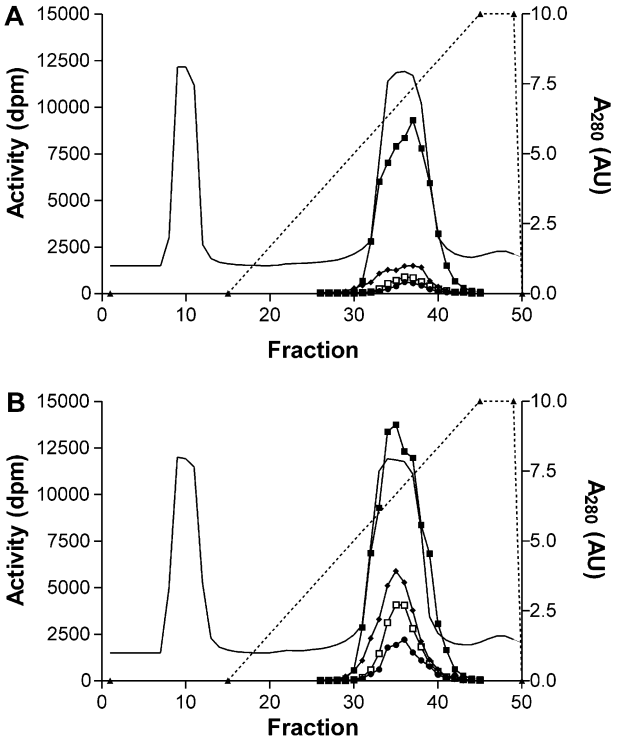

Fig. 4. DEAE anion exchange chromatography of identical amounts of protein from extracts of 10 day wheat shoots which were (A) untreated or (B) treated with cloquintocet mexyl. Proteins were eluted with an increasing concentration of NaCl as shown with the broken line (· · · ·). The eluting proteins were monitored using UV absorbance (A_{280}) as shown (—). Fractions (1 ml) were collected and a 20 μl sample assayed for OGT activity towards 2,4,5-trichlorophenol (■), quercetin (◆), luteolin (●) or coumesterol (□) by radioassay.

1999). It was, therefore, of interest to determine the effect of cloquintocet mexyl and dichlormid on OGT activities in the two black-grass populations (Table 1). In both the wild type and Peldon populations, dichlormid treatment resulted in a minor increase in OGT activity toward luteolin but had no effect on the conjugation of the other substrates. Treatment of the two populations with cloquintocet mexyl decreased OGT activities toward all the substrates tested.

3. Discussion

Wheat is known to *O*-glucosylate a diverse range of phenolics, notably flavonoids (Estiarte et al., 1999), cyclic hydroxamates (Nakagawa et al., 1995), *p*-nitrophenol (Schmitt et al., 1985; Malcherek et al., 1998) and pesticide metabolites derived from the aryloxyphenoxypropionate, sulphonyl urea and phenylurea herbicides (reviewed by Cole and Edwards, 2000). However, relatively little is known about the enzymes which catalyse these conjugation reactions. NGTs with activity toward chloroanilines and OGTs active in conjugating several chlorinated phenols have been described in wheat plants and cell cultures (Schmidt et al., 1985, 1995; Pflugmacher and Sandermann, 1998). Recently, an OGT specific in conjugating cyclic hydroxamates was purified and characterised in wheat seedlings (Sue et al., 2000).

In our studies, we have compared OGT activities toward synthetic and naturally-occurring phenolics in wheat. Under the assay conditions used, although the phenolic substrates were present at saturating concentrations the UDP-[^{14}C-glucose] was used at low concentrations to avoid radioisotope dilution. However, we did ensure with all samples that product formation was directly proportional to protein content. Thus, although the values quoted for enzyme activities underestimate the catalytic efficiency of the OGTs, the results obtained for each substrate are directly comparable between treatments.

The differential inhibition by $MnCl_2$ of OGT activity toward quercetin, but not 2,4,5-trichlorophenol, suggested that distinct OGTs catalyse the conjugation of the flavonol and chlorinated phenol. In view of the large number of GT coding sequences and isoenzymes identified in higher plants this observation is not surprising (Vogt and Jones, 2000). However, the relative specificities of individual plant OGTs for xenobiotic versus endogenous phenolics warrants further study, as the few reports to date are contradictory. In soybean cell cultures, a 47 kDa OGT which showed high activities toward chlorinated phenols,

Table 1
OGT activities towards naturally occurring and synthetic phenolics in the shoots of (A) the herbicide-sensitive wild type and (B) herbicide-resistant Peldon black-grass treated with the safeners dichlormid and cloquintocet mexyl. All values are means ± variation in duplicated determinations after correcting for background conjugation rates

	O-GT activity pmol product min^{-1} mg^{-1} protein		
Substrate	Control	Dichlormid treated	Cloquincet mexyl treated
A			
4-Nitrophenol	0.34 ± 0.01	0.23 ± 0.01	0.13 ± 0.23
2,4,5-TCP	2.67 ± 0.02	2.63 ± 0.16	1.14 ± 0.24
Quercetin	1.14 ± 0.24	4.48 ± 0.27	2.82 ± 0.01
Luteolin	0.52 ± 0.09	0.97 ± 0.00	0.40 ± 0.44
Genistein	0.07 ± 0.00	0.07 ± 0.01	0.11 ± 0.13
Coumesterol	0.16 ± 0.01	0.24 ± 0.01	0.13 ± 0.02
B			
4-Nitrophenol	1.01 ± 0.14	1.06 ± 0.05	0.94 ± 0.23
2,4,5-TCP	8.01 ± 0.53	10.10 ± 0.62	7.54 ± 0.24
Quercetin	8.80 ± 0.38	9.72 ± 0.12	8.06 ± 0.01
Luteolin	1.86 ± 0.09	2.83 ± 0.02	1.09 ± 0.44
Genistein	0.60 ± 0.10	0.96 ± 0.22	0.53 ± 0.13
Coumesterol	0.96 ± 0.04	0.83 ± 0.08	0.52 ± 0.02

including 2,4,5-trichlorophenol, but negligible activity toward flavonoid and phenylpropanoid natural products was purified 1000-fold (Sandermann et al., 1991). In a separate study, a 50 kDa soybean OGT active in conjugating 2,2-*bis*-(4-chlorophenyl) acetic acid (DDA) was purified 367 fold from cell cultures (Wetzel and Sandermann, 1994). This OGT also showed limited activity towards quercetin and naturally occurring benzoic acids. It has also been reported that two soybean OGTs with non-overlapping activities toward kaempferol and 4-hydroxyphenylpyruvic acid respectively, both had activities toward 6-hydroxybentazone, a primary metabolite of the herbicide bentazone (Leah et al., 1992). It will now be of interest to identify the OGTs in wheat with activity toward chlorinated phenols and determine their natural substrate preferences.

Our studies with cloquintocet mexyl and dichlormid demonstrate that OGTs in wheat can be enhanced following safener treatment. This enhancement was dependent on the OGT substrate, the safener used and the organ assayed. Cloquintocet mexyl, a safener used exclusively in wheat, was a more effective inducer of OGT activity than the maize safener dichlormid. Significantly, it was reported that cloquintocet mexyl accelerated the rate of conjugation of a pyridinyl-ring hydroxylated derivative of the herbicide clodinafop *in planta* (Kreuz et al., 1991). Similarly, treatments with the safener BAS 145138 enhanced the rate of glycosylation of an hydroxylated metabolite of chlorimuron ethyl in the roots of maize (Lamoureux and Rusness, 1991). The selective chemical enhancement of the glycosylation of pesticide metabolites has also been reported in potato, where treatment of tuber tissue with (4-chloro-2-methylphenoxy)acetic acid (MCPA) increased the rate of glucose esterification of MCPA, while having no effect on the ether glucosylation of hydroxylated MCPA (Cole and Longhman, 1985). Reports of the enhancement of xenobiotic detoxifying enzymes in wheat have previously been restricted to the safener-induction of GSTs, CYPs and ATP-binding cassette transporters involved in vacuolar sequestration of conjugated pesticide metabolites (Davies and Caseley, 1999; Edwards and Dixon, 2000). The additional safener-enhancement of OGTs suggests that there are common signalling pathways controlling the inducibility of all multiple classes of detoxifying proteins. However, our studies suggest that these signalling pathways must also differ subtly. In wheat shoots, dichlormid enhanced GST expression but not OGT activities, whereas cloquintocet mexyl enhanced both types of conjugating enzymes. In common with GSTs, the safeners selectively increased a sub-set of OGT activities. In the case of wheat GSTs, it is known that the differential enhancement of detoxification activities by safeners (Edwards and Cole, 1996), is due to the selective induction of distinct GST isoenzymes (Cummins et al., 1997; Riechers et al., 1997; Pascal et al., 1998). Our results would suggest that safeners similarly selectively enhance OGT isoenzymes.

As compared with herbicide-sensitive black-grass, the plants from the Peldon population, which show cross-resistance to aryloxyphenoxypropionate and phenylurea herbicides (Hall et al., 1997), contained higher levels of OGT activity toward all substrates tested. Such differences may arise from natural variations in OGT activities in different populations of this non-domesticated grass weed. However, it is significant that, relative to herbicide-sensitive black-grass, Peldon also contains elevated levels of GSTs and CYPs involved in herbicide detoxification (Hyde et al., 1996; Cummins et al., 1999). This would suggest that herbicide cross-resistance in black-grass is associated with the increased expression of CYPs involved in phase I xenobiotic detoxification as

well as two classes of phase II conjugating enzymes. Such enhancement is consistent with a mutation in gene(s) which regulate the co-ordinated expression of CYPs, GSTs and OGTs and has interesting parallels with the induction of these detoxification systems by safeners in wheat. However, OGT activities were unresponsive, or even inhibited by safener applications in both populations of black-grass suggesting that the perturbed regulatory system, which gives rise to herbicide cross-resistance, is distinct from that activated by these safeners.

4. Experimental

4.1. Plant studies

Seeds of bread wheat (*Triticum aestivum* L. cv Hunter) were obtained from Plant Breeding International (Cambridge, UK) and the herbicide-sensitive and herbicide resistant (Peldon) seeds of black-grass (*Alopecurus myosuroides* L.) from Herbiseed (Wokingham, UK). The herbicide safeners dichlormid and cloquintocet mexyl were obtained from British Greyhound (Birkenhead, Merseyside, UK) and Aventis Crop Science respectively. The safeners were prepared as 10 mg ml^{-1} solutions in acetone and were diluted 1000-fold with water prior to plant treatment. Control treatments consisted of 0.1% v/v acetone. The wheat seeds were imbibed in either the safener or control treatments for 1 h prior to sowing on vermiculite. The seeds were then germinated at 25 °C with 8 h dark / 16 h light at a light intensity of 110 μE m^{-2} s^{-1} and kept moist with continued wetting with either control or safener treatments respectively. The black-grass seeds were first placed on wet filter paper on the open bench for 3 days, prior to sowing on Levington compost. The black-grass was then maintained as described for the wheat. At harvest, plants were separated into roots and shoots, weighed and then frozen in liquid N_2 prior to storage at –80 °C.

4.2. Analysis of OGT activities

Frozen tissue was ground to a powder with a pestle and mortar and extracted on ice with 3 v/w of 0.2 M Tris–HCl pH 8.0 containing 2 mM dithiothreitol (DTT) and 5% (w/v) polyvinylpolypyrrolidone. After straining through miracloth, the suspension was centrifuged (8500 *g*, 30 min, 4 °C) and the supernatant decanted prior to the addition of $(NH_4)_2SO_4$ to 70% saturation. Protein pellets were recovered by centrifugation (10,000 *g*, 30 min, 4 °C) and desalted in 0.2 M Tris–HCl pH 8.0 containing 2 mM DTT using Sephadex G-25 gel filtration chromatography (PD-10 columns, Pharmacia). Protein content was determined using dye binding assay reagent with γ-globulin as reference protein as recommended by the manufacturer (BioRad). The protein sample (65 μl) was added to 5 μl of a methanolic solution of the co-substrate (1 mM) and the reaction contents pre-incubated for 3 min at 30 °C. The reaction was initiated with the addition of UDP-[U-^{14}C-glucose] (833 Bq, 7.6 GBq $mmol^{-1}$, ICN) and after incubating for 20 min at 30 °C terminated with the addition of 0.3 M HCl (125 μl). Radioactive conjugates were then quantified by partitioning with water-saturated ethyl acetate (200 μl) and radioassaying 100 μl of the organic phase by liquid scintillation counting (Parry and Edwards, 1994). Reaction products were analysed by radio-HPLC as described previously (Parry and Edwards, 1994). Enzyme activity was expressed as pmol min^{-1} mg^{-1} protein. Control incubation consisted of the enzyme preparation and UDP-[^{14}C-glucose] incubated with 5 μl methanol to correct for the conjugation of endogenous metabolites.

4.3. Immunoblotting and assay of glutathione transferases

Crude protein samples prepared for OGT assay were denatured and applied to 12% SDS-PAGE gels (Cummins et al., 1997). The GST polypeptides present were identified by electroblotting onto a PVDF membrane and probing with anti-GST-sera as described previously (Dixon et al., 1998). Two rabbit antisera were used, one raised to the safener-inducible phi class maize GST *Zm*GSTF1-2 (Dixon et al., 1998) and the other to the constitutive tau class wheat *Ta*GSTU1-1 (Cummins et al., 1997). Antibody-GST complexes were visualised using a secondary anti-rabbit IgG serum coupled to alkaline phosphatase (Dixon et al., 1998). Samples used in immunoblotting experiments were also assayed for GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Dixon et al., 1998).

4.4. Partial purification of OGT isoenzymes

The protein pellets prepared by $(NH_4)_2SO_4$ prepared from wheat foliage were desalted in 25 mM imidazole pH 7.5 containing 2 mM DTT (buffer A) using a Sephadex G-25 column. Protein (total 120 mg) was applied to a 5 ml DEAE column pre-equilibrated in buffer A. After washing the column with 12 ml of buffer A at 1.0 ml min^{-1}, protein was eluted with a linearly increasing concentration of NaCl up to 0.4 M NaCl over 30 min. Fractions (1 ml) were collected and assayed for OGT activity.

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